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**COMPARISON OF THE PROPERTIES  
OF NATIVE AND PENTAAMMINERUTHENIUM(III)-MODIFIED XYLANASE**

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Two xylanases, xynA of *Bacillus pumilus* and xyn II of *Trichoderma reesei*, were purified, then modified by the attachment of pentaammineruthenium, resulting in the generation of a xylanase with veratryl alcohol oxidase activity. Hydrolytic activity of *T. reesei* xyn II on soluble xylans was unchanged by modification with pentaammineruthenium; however, modification of *B. pumilus* xynA greatly reduced xylan hydrolysis unless the active site of the xylanase was protected with xylose during the modification. The presence of histidine, cysteine, or reduced glutathione during xylan hydrolysis greatly increased the xylanase activity of the pentaammineruthenium-modified *B. pumilus* xylanase. Glycine, glutamic acid, methionine, or oxidized glutathione had no effect on xylanase activity.

keywords:

chemical modification/xylanase/ruthenium/oxidase

## Introduction

Ruthenium complexes generate hydrogen peroxide, superoxide, and hydroxyl radicals by reaction with molecular oxygen.<sup>1,2</sup> Pentaammineruthenium can be attached to proteins by coordination of the metal to a surface-accessible histidine residue of the protein as a sixth ligand. Attachment of pentaammineruthenium(III) imparts unique redox properties to myoglobin, azurin, and cytochrome c without perturbing their structure significantly.<sup>3-5</sup> Attachment of pentaammineruthenium to cellobiohydrolase I and II (CBH I and II), the main cellulases of the fungus *Trichoderma reesei*, enables the enzymes to oxidize veratryl alcohol, a substrate of lignin peroxidase, an oxidoreductase activity not present in the native cellobiohydrolases.<sup>6-8</sup>

Xylanases are used to decrease the amount of chemicals required in the bleaching of wood pulp for paper manufacture. Improved efficacy of xylanase pretreatment would further decrease the use of bleaching chemicals and improve the results from "totally chlorine-free" bleaching. Hardwood xylans, such as birchwood xylan, are composed of acetyl-4-O-methylglucuronoxylan with a degree of polymerization (DP) of about 200, and with one in ten of the  $\beta$ -D-xylopyranoside backbone units having a 1,2-linked 4-O-methylglucuronic acid moiety. The xylan found in cereals, such as oat spelt xylan, and other grasses is arabino-4-methyl-glucuronoxylan with a DP of about 70 and with L-arabinofuranosyl side chains.<sup>9</sup> The differences between the two types of xylan can affect the bleaching of different wood pulps.<sup>10</sup> The xylanase pretreatment aids the removal of

lignin from the wood pulp by hydrolyzing the xylan backbone of the hemicellulose to which the lignin is covalently attached. If the xylanase were equipped with a redox group, the bifunctional enzyme thus designed might be able to directly attack and depolymerize the lignin in a manner similar to that employed by lignin peroxidases. In order to design a bifunctional xylanase, we modified two xylanases, xynA from *Bacillus pumilus* and xyn II from *Trichoderma reesei*, by attachment of pentaammineruthenium groups. Both of these xylanases are small proteins with alkaline pI (molecular weight 22,000 and 21,000, and pIs 9.3 and 9.0, respectively). They belong to the G family of xylanases, a group of homologous small xylanases of alkaline pI that have been isolated from various fungi and bacteria.<sup>9,11</sup> The bacterial xylanase, xynA, possesses four histidine residues, at positions 11, 59, 60, and 160 from the amino terminus of the protein; it also has one cysteine residue at position 82. The fungal xylanase xyn II has histidines at positions 22, 144, and 155 from the amino terminus. The catalytically active residues of these xylanases had been reported to be two glutamic acids, at positions 93 and 182 for xynA,<sup>12</sup> and at 86 and 177 for xyn II.<sup>13</sup> Histidine had not been reported to be a catalytically active residue in xylanases,<sup>14-17</sup> so no loss of hydrolytic activity due to attachment of pentaammineruthenium was anticipated. The properties of the modified xylanases are reported here.

## Materials and methods

### *Purification and modification of enzymes*

Two xylanases, xynA of *Bacillus pumilus* and xyn II of *Trichoderma reesei*, were purified from commercially available crude culture filtrates (Pulpzyme HB™ and SP431, formerly Pulpzyme HA™, generous gifts from NovoNordisk Biotech, Inc., Danbury, CT).

Purification stages of the proteins were analyzed by sodium dodecyl sulfate (SDS) and isoelectric focusing polyacrylamide gels.

To purify the *B. pumilus* xylanase, 10-ml aliquots of culture filtrate were applied to 5 × 50 cm columns of Biogel P-30 (BioRad) in 5 mM ammonium carbonate, pH 7.8. Protein fractions from the P-30 chromatography were applied to 1.0 × 9 cm columns of DEAE Sephadex in the same buffer. The purified xylanase appeared homogeneous on SDS gels (Figure 1). Xylanase samples were lyophilized, and the dry protein was used for the modification with pentaammineruthenium(II).

The *Trichoderma* culture filtrate was found to contain all of the *Trichoderma* cellulases, in addition to the two xylanases. The crude filtrate (30 ml) that had been dialyzed overnight at 4°C against 5 mM ammonium carbonate, pH 7.8, was applied to a 50-ml column of DEAE Sephadex in 5 mM ammonium carbonate, pH 7.8. At this pH, xylanase II, with a pI of 9.0, should not bind to the ion-exchange matrix, whereas the cellulase components should bind to the DEAE Sephadex. About 5-10% cellulase (cellobiohydrolase II, endoglucanases I and II) was present in the eluted xylanase II fraction.

Attachment of pentaammineruthenium to the xylanases was carried out as described previously for CBH I.<sup>6</sup>

### *Analytical methods*

The presence of pentaammineruthenium attached to histidine was determined spectrophotometrically by the appearance of an absorption peak at 360 nm and by the appearance of a peak at 110 mV in square-wave voltammograms.<sup>18</sup> The ratio of Ru(NH<sub>3</sub>)<sub>5</sub>-histidine to xylanase was calculated from the absorbance of the modified xylanase at 360 nm and the molar absorption coefficient of Ru(NH<sub>3</sub>)<sub>5</sub>-histidine (3000 M<sup>-1</sup> cm<sup>-1</sup>), and from the absorbance of the protein at 280 nm, using a molar absorption coefficient (56,000 M<sup>-1</sup> cm<sup>-1</sup>) that was calculated from the tryptophan and tyrosine content of the protein.<sup>12</sup>

Polyacrylamide gel electrophoresis was carried out with a Pharmacia Biotech Phast™ system using Pharmacia Biotech precast IEF 3-9 and 10-15 gradient gels. Pharmacia Biotech low-molecular-weight markers and IEF 3-10 markers were prepared and used according to the manufacturer's instructions. Samples for SDS electrophoresis were incubated with 2% SDS and 5% β-mercaptoethanol for 5 min at 90°C before loading them on the gels.

### *Assays of enzymatic activity*

Oxidoreductase activity was assayed with the lignin peroxidase substrate veratryl alcohol.<sup>7,19</sup> Xylanase assays were carried out at 45°C in 50 mM sodium acetate, pH 6.0 (pH 5.0 for the *Trichoderma* xylanase) with 1% birchwood or oat spelt xylan and 0.010

mg/ml xylanase. Xylanase activity was assayed by measuring reducing sugar production with the dinitrosalicylic acid reagent.<sup>20</sup> In determining the optimal pH for the *B. pumilus* enzyme, 50 mM sodium phosphate buffer was used for the assays at pH 7.0, and 50 mM Tris-HCl buffer was used for the assays at pH 8.0, 8.5, or 9.0.

#### *High-pressure liquid chromatography (HPLC) analysis*

Purification of xylanase samples prior to tryptic digestion as well as fractionation of tryptic peptides was carried out with a Perkin-Elmer Applied Biosystems Model 172 microbore liquid chromatography system. For reverse-phase chromatography, a 1.0 × 100 mm Nucleosil C18 column and a 0.5 × 150 mm Reliasil C18 column (Column Engineering, Ontario, CA) were run using a gradient of 2% acetonitrile/0.05% trifluoroacetic acid to 90% acetonitrile/0.045% trifluoroacetic acid, with a flow rate of 0.025 or 0.06 ml/min, depending on column diameter. Whole xylanase was detected by monitoring at 280 nm; tryptic peptides, by monitoring at 216 and 305 nm.

#### *Tryptic digests*

Samples (1.0 mg/ml) of native and modified xylanase were purified by C18 reverse-phase HPLC before digestion with trypsin. Purified xylanase was dried and then redissolved in 0.020 ml of 0.2 M ammonium bicarbonate, pH 8.5, to a final reaction



volume of 0.030 ml. Then 2  $\mu$ l of a solution of 1 mg/ml modified trypsin (Promega) was added, and digestion was allowed to proceed for 12 h at ambient temperature. Digestion was terminated by the addition of 0.005 ml trifluoroacetic acid, followed by drying the sample *in vacuo*.

### *Mass spectrometry and peptide sequencing*

Analysis by mass spectrometry and peptide sequencing was carried out at the Protein and Peptide Micro Analytical Facility, which is located at the Beckman Institute, the California Institute of Technology, Pasadena, California.

Matrix-assisted, time-of-flight (MALDI TOF) mass spectrometry was carried out with a Lasertech II mass spectrometer (Perseptive Biosystems, Cambridge, MA), equipped with a reflector, a 1.3-m flight tube, and a nitrogen laser. HPLC fractions (0.5  $\mu$ l) were spotted to the sample slides with sinapinic acid (10 mg/ml) and  $\alpha$ -cyano-4-hydroxycinnamic acid. Acetylangiotensinogen (MW 1801.09) and bovine cytochrome c (MW 12,360.1) were used as internal and external calibrants.

Peptides were identified by Edman degradation with a model 476 automated microsequencer (Perkin Elmer/Applied Biosystems, Foster City, CA) and comparison with the known sequence. The coupling buffer was N-methyl-piperidine, and the samples were spotted to Porton peptide disks (Porton Instruments Division, Beckman Instruments,

Oxnard, CA).

## Results

### *Purification of xylanases*

The bacterial xylanase xynA was reportedly produced in a bacterial strain without endogenous cellulases and was apparently homogenous, simplifying its purification. The majority of the modification and pulping experiments were therefore carried out with this xylanase. Contaminants from the culture media were removed by gel filtration on Biogel P-30 in 5 mM ammonium carbonate, pH 7.8, followed by ion-exchange chromatography on DEAE Sephadex in the same buffer (Figure 1).

In the case of the *T. reesei* xylanase xyn II,<sup>13</sup> the culture filtrate obtained from NovoNordisk contained the entire multicomponent cellulase system of the fungus with xyn II making up only about 10% of total protein. The relative amount of xylanase is increased, but the cellulases are still present in large amounts,<sup>21</sup> necessitating purification with multiple ion-exchange chromatography steps. To evaluate the suitability of this enzyme, modification of this partially purified xylanase preparation was carried out. The attachment of pentaammineruthenium to xyn II did not adversely affect its xylanase activity to the same degree as observed in the case of the bacterial xylanase described above. The hydrolytic activity of pentaammineruthenium-modified xyn II towards birchwood and oat

spelt xylans remained fairly close to that of the native control (modified, 162 and 168  $\mu\text{mol mg}^{-1} \text{ min}^{-1}$ ; native, 207 and 190  $\mu\text{mol mg}^{-1} \text{ min}^{-1}$ ).

*Properties of pentaammineruthenium(III)-modified xynA*

The effect of modification with pentaammineruthenium upon the xylanase xynA from *Bacillus pumilus* depended upon the modification conditions. Modification was carried out for 2 or 4 h with a tenfold excess of aquopentaammineruthenium(II). The properties of the xylanase after attachment of pentaammineruthenium were the same for the 2- and 4-h modification times, but attachment was found to be more reliable when a 4-h modification time was used. A substantial loss of activity in hydrolysis of birchwood and oat spelt xylans was observed for xynA modified with pentaammineruthenium. Loss of activity was reduced when xynA was modified in the presence of 0.10 M xylose to protect the active site of the enzyme (Table 1). Veratryl alcohol oxidase activity was higher for xynA modified without xylose, and the number of pentaammineruthenium groups attached to the xylanase, based on the absorption peak at 360 nm from the pentaammineruthenium-histidine, was 2 to 3, as opposed to 1 for enzyme protected with xylose.

The optimal pH for hydrolysis of birchwood xylan was not changed by the attachment of pentaammineruthenium. For both native and modified xynA, maximum hydrolysis was observed at pH 6.0 (Figure 2).

*Effect of free amino acids on xylanase activity*

When the inactivated xylanase was incubated with 50 mM histidine at pH 6.0, activity towards xylan was partially restored. No restoration of activity occurred when the modified xylanase was incubated with 50 mM glycine, implying that the imidazole group of the histidine was required for restoration of activity (Figure 3). When the xylanase was incubated with 200 mM histidine for 18 h at 4°C and then filtered on Sephadex G-25 to remove excess histidine, the absorption peak at 360 nm from  $\text{Ru}(\text{NH}_3)_5$ -histidine disappeared (data not shown). Since the absorption peak at 360 nm is characteristic of pentaammineruthenium-histidine, its disappearance implies that the ruthenium complex was displaced from the histidine on the xylanase by the excess free histidine. Further investigations of the effects of various amino acids upon the xylanase activity of inactivated, pentaammineruthenium-modified xynA are summarized in Table 2. Amino acids were added directly to control and enzyme reaction mixtures containing 1% birchwood xylan, and the net production of reducing sugar was determined for the reaction containing enzyme. At the concentrations used in this study (5-50 mM), histidine doubled the specific activity of the inactivated xylanase, but reduced glutathione and cysteine increased it fourfold to fivefold. Oxidized glutathione, glutamic acid, glycine, and methionine had no effect on the activity of the xylanase (Figure 3 and Table 2). Control reactions in which xylan was omitted showed no product from the action of the modified xylanase on cysteine, or on reduced or oxidized glutathione, which gave a reaction with the

dinitrosalicylic acid reducing sugar reagent.

*Determination of site of attachment of pentaammineruthenium(III)*

To determine the site of attachment of the pentaammineruthenium on the modified xynA, Edman sequence determination and mass spectroscopy were carried out in tandem on whole native and modified xynA and on tryptic peptides prepared from the two proteins.

Before further analysis by mass spectroscopy and tryptic digestion was carried out, native and pentaammineruthenium(III)-modified xynA were purified by reverse-phase HPLC on Nucleosil C18. Mass spectroscopy of the HPLC-purified proteins gave a mass value for native, unmodified xylanase of  $22,577 \pm 25$  amu and a mass value for pentaammineruthenium(III)-modified xynA of  $22,950 \pm 50$  amu. The increase in mass of 370 amu observed for modified xylanase corresponds to that expected for the addition of two pentaammineruthenium groups (mass 186) to the protein.

Native and pentaammineruthenium(III)-modified xynA were sequenced directly from the amino terminus using Edman degradation. Peptide sequencing of the whole protein of pentaammineruthenium(III)-modified xynA identified the first 20 amino acid residues and determined that the ruthenium complex had not been attached to H11. The N-terminal amino acid sequence was observed to differ from the published sequence of xynA from *Bacillus pumilus*:

published sequence: RTITNNEMGNHSGYDYELWKD

observed sequence: ETIYDNRIGTHSGYDFELWKD

Another six differences between the published sequence and the observed sequence were noted: N44 was changed to K, A159 was changed to E, R161 was missing, M173 was changed to S, Q184 was changed to S, and S186 was changed to K. Overall, the percent identity between the published sequence<sup>17</sup> and the sequence determined from the NovoNordisk Pulpzyme HB™ xylanase was 93%. It appears that the NovoNordisk enzyme may be isolated from a different strain of *Bacillus pumilus* than that used in the published sequence determination.

The tryptic peptides generated from native and modified xylanase were compared by analysis with HPLC and mass spectroscopy, a method that has been used to determine the site of attachment of substrate analogs to exoglucanases and  $\beta$ -glucosidases.<sup>22</sup> Tryptic digests were carried out on native and modified xynA as described above. The tryptic peptides were fractionated by reverse-phase HPLC. The HPLC effluent was monitored at 216 nm to detect peptides and at 305 nm to detect the presence of pentaammineruthenium-histidine (absorbance maximum is shifted to 305 nm at acid pH). When detection was carried out at 216 nm, a peak present in the column profile of the tryptic digest of native xylanase at 32.5 min was absent from the chromatogram of the pentaammineruthenium(III)-modified xylanase (Figure 5). This peak, missing from the tryptic digest of the modified xylanase, was found to contain several peptides, including one with a mass of 1279.5 amu. The peptides present in the peak were sequenced, and the peptide of mass 1279.5 was determined to correspond to residues 151-162 of the published sequence.

To establish that the peptide corresponding to 151-162 of the published sequence was the site of attachment of pentaammineruthenium, the chromatogram of the tryptic peptides from the modified xylanase was examined for the presence of a peptide corresponding to the mass of the missing peptide plus one or two pentaammineruthenium groups. When the chromatogram of the tryptic digest of modified xylanase was monitored at 305 nm, several peaks were detected. One peak, which eluted at 31 min in the HPLC chromatogram, had a high ratio of absorbance at 305 nm to absorbance at 216 nm, indicating that it was likely to contain pentaammineruthenium-modified peptides. The presence of a peptide with mass of 1650.8 was detected in the peak at 31 min from the tryptic digest of the modified xylanase. The mass of this peptide (1279.5 amu) corresponds to the mass of the native peptide consisting of residues 151-162, plus a mass equivalent to two pentaammineruthenium groups. Sequence determination by Edman degradation confirmed the identity of the peptide present in the peak eluting at 31 min as corresponding to residues 151-162 of the xylanase:

TSGTVSVSEHFK

No tryptic peptides of mass 1650.8 were detected in the digest of the native xylanase. This implies that the pentaammineruthenium is attached to the 151-162 peptide, which contains one histidine at position 160. The location of the attached pentaammineruthenium thus appears, by a process of elimination, to be the histidine at position 160, an amino acid residue that is found to be conserved among the family G xylanases.<sup>13</sup> The second pentaammineruthenium group could be attached to the glutamic acid residue adjacent to the

histidine on the same peptide.

*Determination of kinetic parameters for native and pentaammineruthenium-modified xynA*

To investigate the effect of attachment of pentaammineruthenium to xynA, the kinetic parameters of native and 4-h pentaammineruthenium-modified xynA were determined for the hydrolysis of birchwood xylan (Figure 6 A and B). The  $K_m$  values for native and modified xynA were determined to be 6.44 and 3.76 mg/ml birchwood xylan, respectively. For native and modified xynA, the  $V_{max}$  values were 464 and 141 mM reducing sugar  $\text{min}^{-1}$  (mg xylanase) $^{-1}$ , respectively. Loss of activity upon modification would thus appear to result from the inability of the modified xylanase to carry out catalysis, since the  $V_{max}$  is decreased fourfold compared with the native enzyme. The decrease in  $K_m$  upon modification could be the result of a conformational change in the active site caused by attachment of the pentaammineruthenium groups.

## **Discussion**

Initial studies have shown that oxidoreductase activity can be conferred upon xylanases by attachment of pentaammineruthenium groups. The specific activity of the



modified xylanases towards the lignin monomeric substrate veratryl alcohol increases with the number of pentaammineruthenium groups attached per molecule of xylanase.

The loss of xylanase activity observed due to modification of the *B. pumilus* xylanase was unexpected, as histidine had not been reported to be a catalytically active residue in xylanases. Two conserved glutamic acid residues had been reported to be essential for catalysis of xylan hydrolysis in a number of xylanases.<sup>14-17</sup> A comparison of the amino acid sequences of the different xylanases indicates that three histidine residues (positions 11, 59, and 60) are present in xynA from *B. pumilus* that are not conserved in several other family G xylanases<sup>11,23</sup> isolated from bacterial and fungal sources, including *T. reesei* xyn II. The histidine at position 159 in the *B. pumilus* xynA is conserved among ten bacterial and fungal xylanases, including the xylanases from *B. pumilus*, *Bacillus subtilis*, and *T. reesei*.<sup>13</sup> Examination of the three-dimensional structure of the xylanase<sup>24,25</sup> reveals that His-159 is located inside the active site groove, while His-59 and His-60 reside on a loop located below the active site. It is possible that this conserved histidine may help carry out catalysis; thus, its modification results in a loss of hydrolytic activity. Alternatively, attachment of the pentaammineruthenium group to a residue in the active site cleft may obstruct access of the catalytic glutamic acid residues to the substrate. The kinetic analysis of the reaction of native and pentaammineruthenium-modified xynA with birchwood xylan appears to indicate that binding of the enzyme to the substrate is not adversely affected and, in fact, would appear to be better for the modified xynA (an approximately twofold decrease in  $K_M$ ). However,  $V_{max}$  is decreased about fourfold for the

modified xynA, indicating a considerable decrease in the rate of the hydrolysis reaction.

Modification with pentaammineruthenium or other histidine-modifying reagents in the presence or absence of xylose can be used as a probe of the role of histidines in the active site of xylanases. A reversibly inactivated xylanase may be useful for applications requiring strict limitation of the degree of xylan hydrolysis. A method using imidazole instead of histidine, or a sulfhydryl reagent instead of cysteine, could be devised to increase the cost-effectiveness of *in situ* reactivation of pentaammineruthenium-inactivated xylanase.

Treatment of wood pulps with xylanases to reduce the amount of chemicals required for bleaching is a method that is currently being investigated and is of great interest to the pulp and paper industries.<sup>10,26-29</sup> In one experiment, bleaching pretreatment of kraft pulps using xylanases modified with pentaammineruthenium was shown to reduce the requirement for chlorine dioxide to a greater degree than pretreatment with native xylanases (data not shown). Further investigation and application of these preliminary results could, if confirmed, result in improvement of the quality of paper while reducing the requirement for bleaching chemicals, thus decreasing the amount of halogenated organic compounds that would have to be removed from paper mill effluents. Pentaammineruthenium and pentammineruthenium-modified proteins have been reported to produce reactive oxygen species and to carry out oxidation of organic compounds.<sup>1-3</sup> Xylanases with attached redox groups such as pentaammineruthenium could also be used to solubilize pulp residues and depolymerize lignin in the treatment of effluent streams.

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**Table 1.** Comparison of native and 4-h pentaammineruthenium-modified xylanase xynA

Xylanase preparation	Specific activity <sup>a</sup>		
	Birchwood xylan	Oat spelt xylan	Veratryl alcohol
Native enzyme	194 ± 12 (N = 4)	137 ± 11 (N = 5)	ND
Trial 1—Mod.	19.8	14.5	0.0115
Trial 2—Mod.	17.9 ± 0.7 (N = 2)	14.0 ± 13.1 (N = 2)	0.0649
Trial 3—Mod. (+ Xylose)	148 ± 1.7 (N = 2)	86.1 ± 4.2 (N = 3)	0.00938

<sup>a</sup>Specific activities for catalytic activities on the indicated substrates in  $\mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$ .

**Table 2.** Effect of free amino acids on xylanase activity of pentaammineruthenium(III)-modified xynA

Amino acid added	Concentration (mM)	Specific activity <sup>a</sup>
None	0	18.7 ± 2.9
Glycine	50	17.0
Glutamic acid	50	18.1
Histidine	50	37.1
Methionine	50	20.6
Cysteine	5.0	75.1
Cysteine	10	86.0 ± 2.1
Glutathione (Red.)	10	104.1
Glutathione (Oxid.)	10	21.7

<sup>a</sup>Specific activity in  $\mu\text{mol}$  reducing sugar produced  $(\text{mg xylanase})^{-1} \text{min}^{-1}$  after incubation for 15 min at pH 6.0 and 45°C from the substrate birchwood xylan (1%).

**Figure 1** SDS gel (10-15% gradient) of xynA preparations. Lanes 1 and 6, Pharmacia LMW markers; Lane 2, Biogel P-30 filtered xynA (Pulpzyme HB™); Lane 3, xynA after Biogel P-30 filtration/DEAE Sephadex ion-exchange chromatography; Lane 4, xynA modified with 50-fold excess pentaammineruthenium for 2 h; Lane 5, xynA modified with 50-fold excess pentaammineruthenium for 4 h. Protein samples were denatured by incubation with 2.5% SDS and 5%  $\beta$ -mercaptoethanol for 5 min at 90°C.

**Figure 2** Effect of pH on hydrolysis of birchwood xylan by native and pentaammineruthenium- modified xynA. The amount of reducing sugar produced by a reaction mixture containing 0.020 mg/ml xylanase, 1% birchwood xylan, and 50 mM buffer was measured after 15-min incubation at 45°C. Native xynA control = ○; xynA modified for 4 h with pentaammineruthenium = ●.

**Figure 3** Effect of free amino acids on xylanase activity of 4-h pentaammineruthenium-modified xynA. Native xynA control = ○. Pentaammineruthenium-modified xynA was incubated with the following amino acids: no amino acids added = ● ; with 10 mM cysteine = ∇; with 10 mM reduced glutathione = ▼ ; with 10 mM oxidized glutathione = □; with 50 mM histidine = ■; with 50 mM glutamic acid = Δ; with 50 mM methionine = ▲; with 50 mM glycine = ◇.

**Figure 4** Chromatograms of the HPLC profiles of tryptic peptides from digestion of native (—) or pentaammineruthenium-modified (- -) xylanase xynA.

**Figure 5 A** Michaelis Menten plot of kinetic data for native and pentaammineruthenium-modified xynA hydrolysis of birchwood xylan. Native xynA = ○; 4-h pentaammineruthenium-modified xynA = ●. **B** Double reciprocal plot of kinetic data for native and pentaammineruthenium-modified xynA. Native xynA = ○; 4-h pentaammineruthenium-modified xynA = ●.

abbreviated running title: Pentaammine-ruthenium-modified xylanase

MW ( $\times 10^{-3}$ )

94

67

42

30

20

14

1

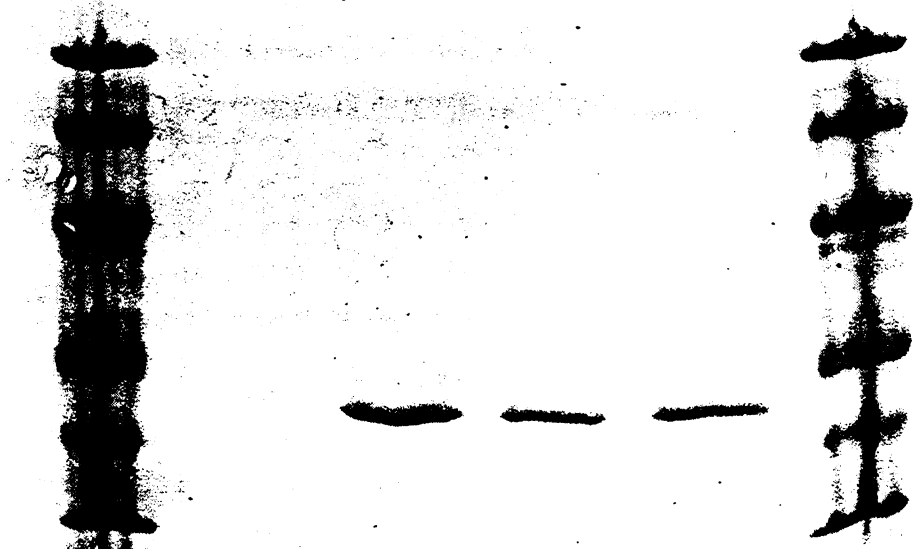
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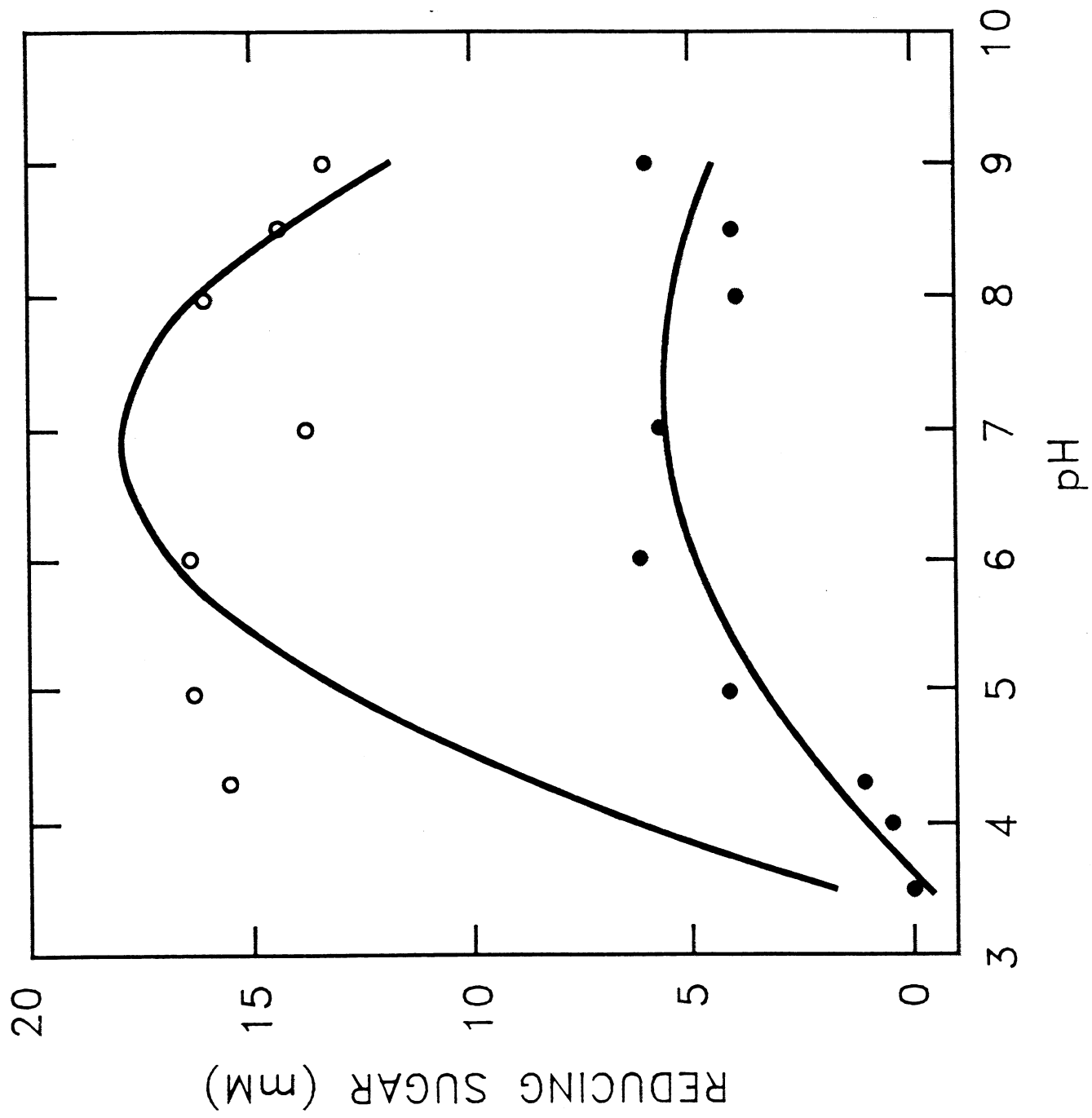
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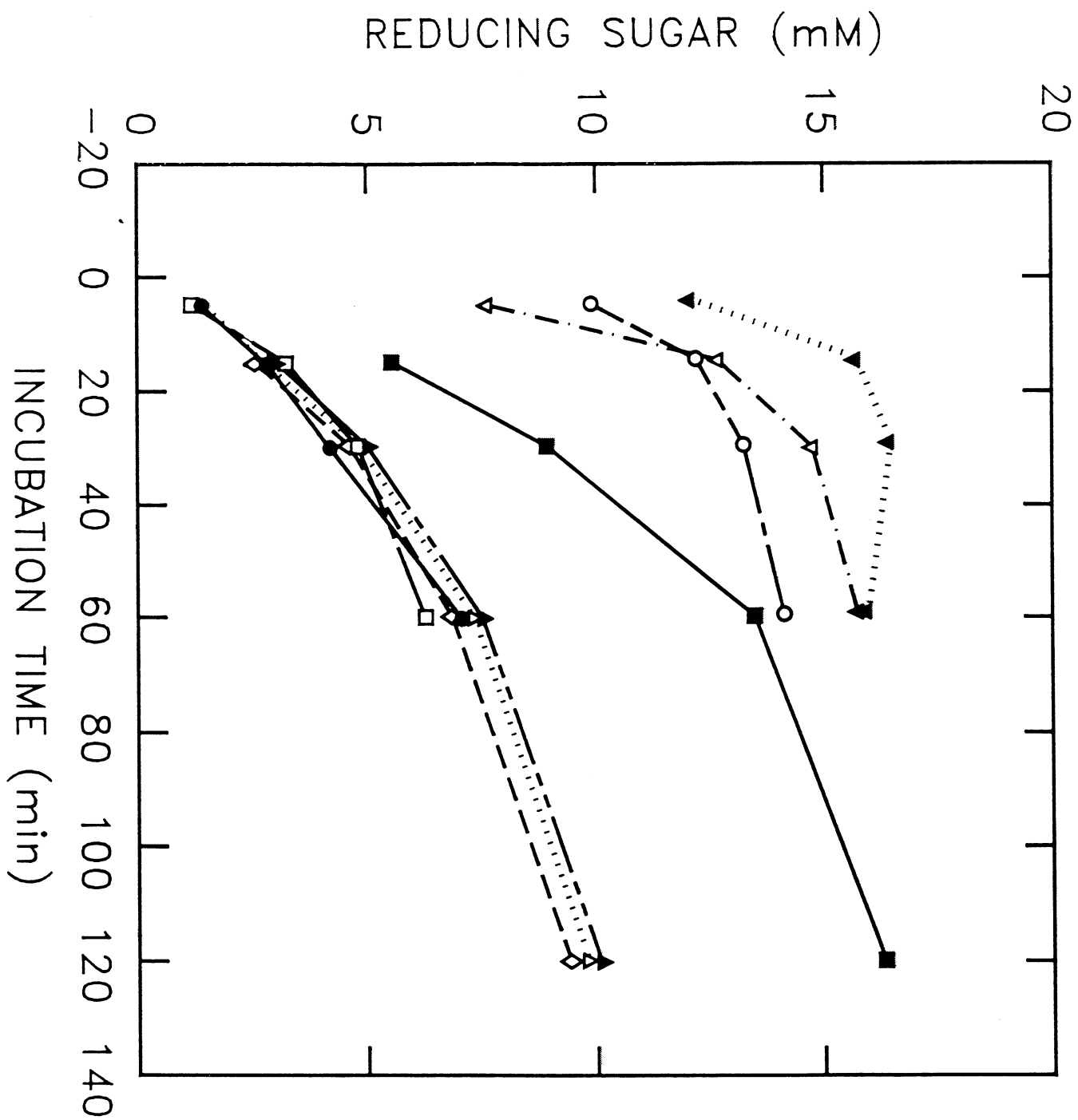
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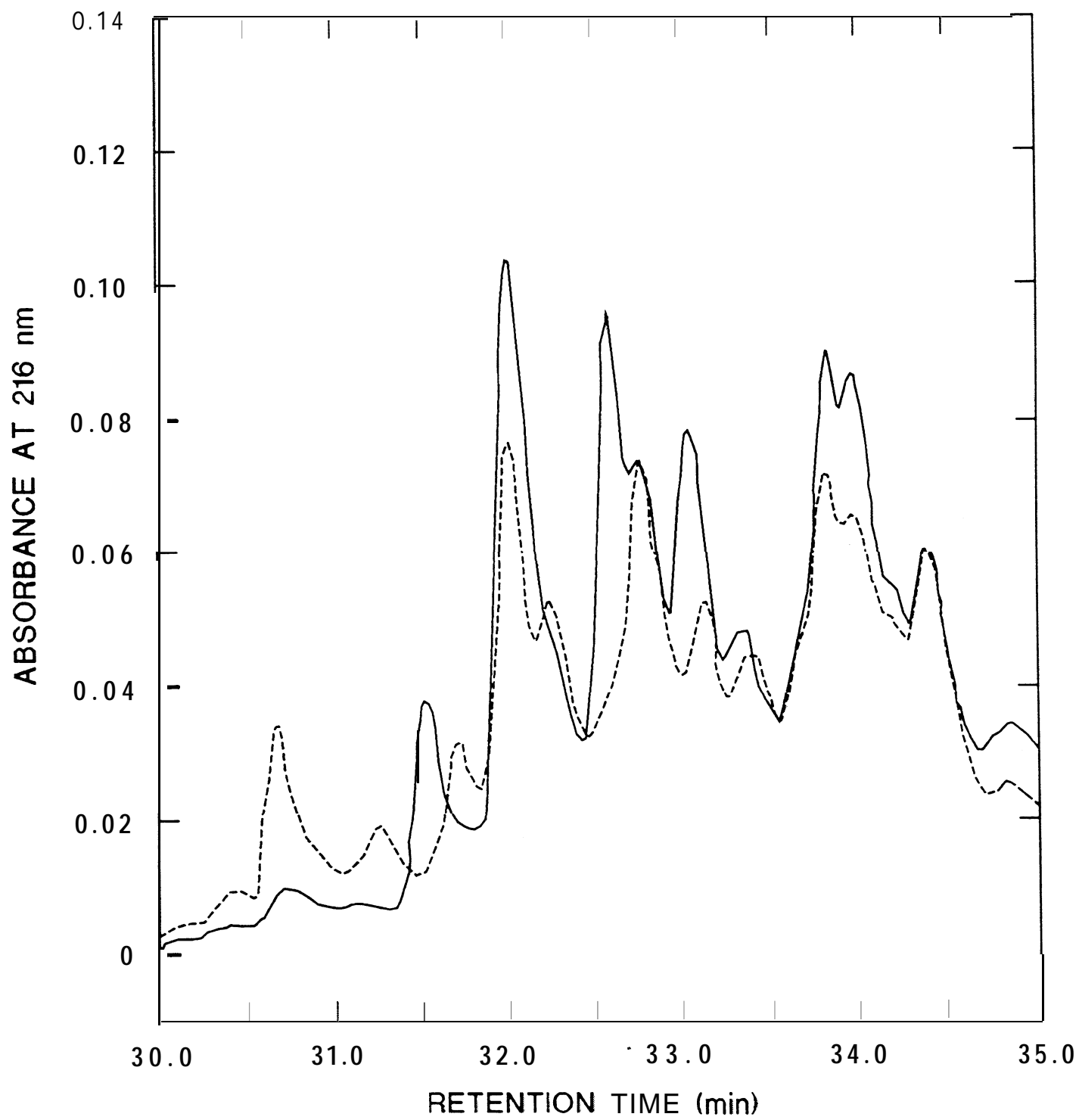


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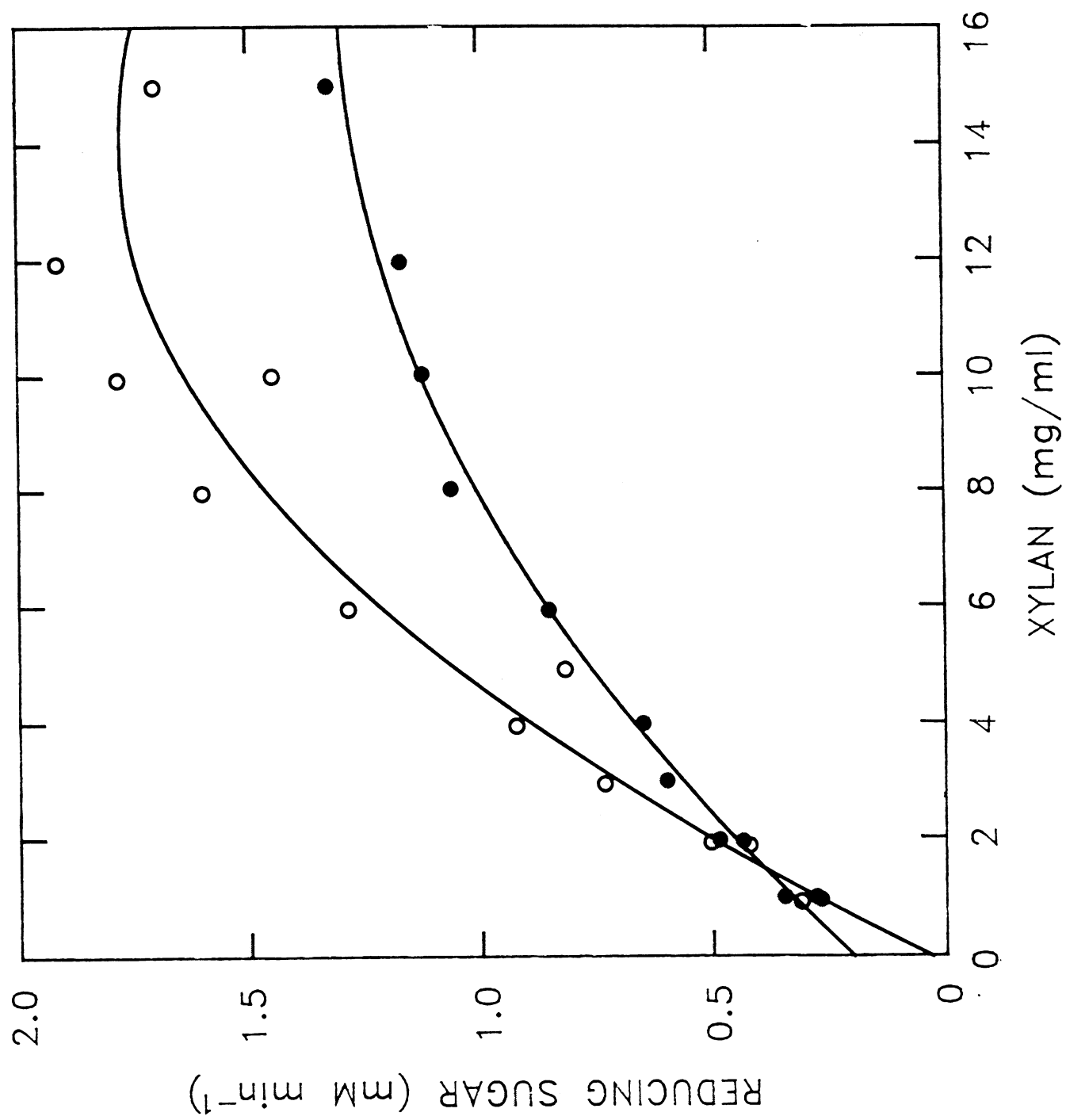








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